Remarks

The Amendments

Claim 1 has been amended to delete the recitations "or a functional equivalent thereof" and "solely." Claim 1 also has been amended to recite that "the heterologous gene expresses an active pyruvate decarboxylase." This amendment is supported by Example 10 of the specification, which discloses expression of an active pyruvate decarboxylase from a heterologous gene.

Claims 9, 12, and 13 have been rewritten as independent claims.

Claim 14 has been amended to recite bacterial strain "LN-DP1" in place of "LN-P1." The amendment corrects an obvious typographical error. See Example 6, which describes the "Production of Strain LN-DP1." (Page 10, line 22 to page 11, line 5.)

New claims 26-28 recite that the bacterium of claims 9, 12, and 13 is a thermophile. These new claims are supported by originally filed claim 3.

New claims 29-31 recite that the bacterium of claims 9, 12, and 13 further comprises an inactivated lactate dehydrogenase gene. New claims 29-31 are supported by originally filed claim 5.

None of these amendments introduces new matter or requires a new search.

Rejection of claims 1-13 under 35 U.S.C. §112, second paragraph

Claims 1-13 are rejected under 35 U.S.C. §112, second paragraph as being indefinite. Applicants respectfully traverse.

The Office Action asserts that the recitation "but has solely native alcohol dehydrogenase function" in claim 1 is indefinite. As suggested in the Office Action, this recitation has been replaced by the recitation "has native alcohol dehydrogenase function."

The Office Action also asserts that the recitation "or a functional equivalent thereof" in claim 1 is indefinite. To advance prosecution, this recitation has been deleted.

Withdrawal of these rejections of claims 1-13 is respectfully requested.

Rejection of claims 1-14 under 35 U.S.C. §112, first paragraph

Claims 1-14 are rejected under 35 U.S.C. §112, first paragraph as lacking both written description and enablement. Applicants respectfully traverse.

Written description rejection of claims 1-14

The Office Action asserts that the broad genus of "a heterologous gene encoding pyruvate decarboxylase (pdc) or a functional equivalent thereof" is not adequately described in the specification. Amended claim 1, however, does not recite this broad genus. To advance prosecution, claim 1 has been amended to recite the genus of "a heterologous gene encoding pyruvate decarboxylase." The specification adequately describes this genus.

Heterologous genes encoding pdc were well known in the art prior to the filing of the application. A heterologous gene is a gene derived from a different species. (See Merriam Webster's definition of "heterologous"; Appendix A.) The specification teaches that heterologous pdc genes "may be from *Zymomonas sp*, preferably *Z. mobilis* or may be from yeast e.g. the *S. cerevisiae* pdc 5 gene." (Page 5, lines 1-2.) The nucleotide sequence of at least ten other pyruvate decarboxylase genes derived from species other than a species of gram-positive bacteria (*i.e.*, heterologous genes) were known in the art prior to the January 6, 2000

effective filing date of the application:

- Accession number X87929 (Appendix B) discloses the nucleotide and amino acid sequence for *Kluyveromyces lactis* pyruvate decarboxylase;
- Accession number X81854 (Appendix C) discloses the nucleotide and amino acid sequence for *Nicotiana tabacum* pyruvate decarboxylase;
- Accession number U71122 (Appendix D) discloses the nucleotide and amino acid sequence for *Arabidopsis thaliana* pyruvate decarboxylase-2;
- Accession number U65927 (Appendix E) discloses the nucleotide and amino acid sequence for *Neospora crassa* pyruvate decarboxylase;
- Accession number X92743 (Appendix F) discloses the nucleotide and amino acid sequence for *Oryza sativa* pyruvate decarboxylase;
- Accession number X59546 (Appendix G) discloses the nucleotide sequence and amino acid sequence for *Zea mays* pyruvate decarboxylase;
- Accession number U75311 (Appendix H) discloses the nucleotide and amino acid sequence for *Pichia stipitis* pyruvate decarboxylase;
- Accession number AF098293 (Appendix I) discloses the nucleotide sequence and amino acid sequence for *Aspergillus oryzae* pyruvate decarboxylase;
- Accession number AI728334 (Appendix J) discloses the nucleotide sequence for *Gossypium hirsutum* pyruvate decarboxylase isozyme 1; and
- Accession number Z54096 (Appendix K) discloses the nucleotide sequence of a cosmid from *Schizosaccharomyces pombe* chromosome 1 that includes the nucleotide and amino acid sequence of pyruvate decarboxylase (page 5).

These sequences were retrieved from the National Center for Biotechnology Information (NCBI) database and bear dates that are at least two and one half months before the priority date of the present application. A specification need not disclose what is well known to those skilled in the art and preferably omits that which is well known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986). Thus, the teachings of the specification,

together with the knowledge in the art of numerous "heterologous genes encoding pyruvate decarboxylase," adequately demonstrates that applicants were in possession of the invention of claims 1-13 when the present specification was filed.

New claims 26-31 should also not be subject to this rejection. They each ultimately depend from claim 1 and thus also a recite gram-positive bacterium transformed with a heterologous gene encoding pdc.

Claim 14 recites particular deposited strains of bacteria. A reference in the specification to a deposit constitutes an adequate description of the deposited material sufficient to comply with the written description requirement. *Enzo Biochem v. Gen-Probe Incorporated*, 296 F.3d 1316, 1325 (Fed. Cir. 2002). Thus claim 14 also is adequately described.

Withdrawal of this rejection of claims 1-14 is respectfully requested.

<u>Enablement rejection of claims 1-14</u>

The Office Action asserts that claims 12-14 are not enabled because they recite novel vectors and strains of microorganisms, some of which have been deposited, but have not been indicated as being publicly available. (Paper 12, page 5, lines 10-16.) Applicants respectfully traverse.

Claim 12 recites a Gram-positive bacterium transformed with a plasmid, pFC1. The specification enables how to make and use plasmid pFC1. The specification discloses that plasmid pFC1 "was formed from a fusion of pAB124 (Bingham et al., Gen. Microbiol., 114, 401-408, 1979) and pUC18." (Page 12, lines 18-20.) The specification also teaches that as a result of fusing pAB124 and pUC18, plasmid pFC1 has a restriction enzyme cleavage map as shown in Figure 8. The plasmid pAB124 was known and used at the time the application was filed. Bingham (*J. Gen. Microbiol. 119*, 109-115, 1980) teaches, at Figure 2, a restriction

enzyme cleavage map of plasmid pAB124 (Page 114; Appendix L.) The sequence the plasmid pUC18 was known at the time the application was filed. GenBank accession number L09136 discloses the sequence of the complementary strand of pUC18. See Appendix M. One of skill in the art would have been able to make and use plasmid pFC1 as shown in Figure 8 using the well known structures of pAB124 and pUC18 and the routinely practiced techniques of molecular biology.

Claims 13 and 14 recite ten strains of bacteria. The recited bacterial strains are LN, LN-T (31), LN-T (32), TN, TN-P1, TN-P3, LN-S (J8), LN-D, LN-D11, and LN-DP1. Four of the recited bacterial strains, LN, TN, LN-S (J8), and LN-D, have been deposited with the National Collections of Industrial, Food and Marine Bacteria (NCIMB). These strains will also be made publicly available upon issuance of a patent. See the declaration of the inventors that accompanies this response. Appendix N. Thus each of these four bacterial strains are enabled.

The specification teaches one of skill in the art how to make and use the remaining six bacterial strains without undue experimentation. The specification teaches how to make and use bacterial strains TN-P1 and TN-P3. The specification discloses that bacterial strain TN-P1 is produced from bacterial strain TN after transformation with plasmid pBST22-zym. (Example 8; page 11, lines 21-24.) The specification discloses that bacterial strain TN-P3 is produced by transforming bacterial strain TN with plasmid pFC-PDC1. (Example 9, page 12, lines 15-18.) Those of skill in the art routinely practice transformation of bacteria. Thus, one of skill in the art could readily produce bacterial strains TN-P1 and TN-P3 using the deposited TN bacterial strain.

The specification also teaches one of skill in the art how to make and use bacterial strains LN-T (E31, E32). The specification teaches that bacterial strains LN-T (E31, E32) are "spontaneous transposon mutants from strain LN. Both strains are lactate deficient." (Page 9,

lines 6-7.) Thus strain LN-T (E31, E32) bacteria are strain LN bacteria that have spontaneously lost LDH activity. One of skill in the art would have been able to produce and identify LN-T strain bacteria by merely culturing the deposited LN strain bacteria and screening the cultured bacteria for LDH activity. Methods of assaying LDH activity of bacteria were also well known at the time the application was filed. Payton *et al.* (FEMS Microbiology Letters, 26, 333-336, 1985; Appendix O) teaches the identification of *Bacillus stearothermophilus* that lack LDH activity. Screening for *B. stearothermophilus* that lacked LDH activity "was achieved by selection for resistance to a suicide substrate, fluoropyruvate." (Page 333, lines 8-9 of the Summary; See also page 334, column 1, line 15 to column 2, line 40.)

The specification also teaches one of skill in the art how to make and use bacterial strain LN-D11 without undue experimentation. The specification discloses that LN-D11 bacteria are produced from strain LN-D bacteria "after repeated subculture." (Page 10, line 13.) LN-D11 bacteria are similar to strain LN-D bacteria except they are sensitive to kanamycin. See Figure 1, which discloses that LN-D strain bacteria are spo^-, ldh^-, km^R, (sporulation deficient, LDH recombination mutant, and kanamycin resistant) and LN-D11 bacteria are spo^- and ldh^- (sporulation deficient and LDH recombinant mutant). One of skill in the art, using the well known techniques of cell culture would have been able to subculture the deposited LN-D strain bacteria and screened it for kanamycin resistance without undue experimentation.

The specification also teaches one of skill in the art how to make and use bacterial strain LN-DP1. The specification discloses that LN-DP1 bacteria are "produced from strain LN-D11 after transformation with the replicative plasmid pBST22-zym." (Page 10, lines 24-25.) One of skill in the art could readily have produced bacterial strain LN-D11 as indicated above. One of skill in the art would further have been able to transform the LN-D11 strain bacteria with

plasmid pBST22-zym by routine experimentation. Thus, one of skill in the art would also have been able make and use bacterial strain LN-DP1 without having to resort to undue experimentation.

Withdrawal of this rejection to claims 12-14 is respectfully requested.

Rejection of claims 1, 2, 8, and 11 under 35 U.S.C. §102(b)

Claims 1, 2, 8, and 11 are rejected under 35 U.S.C. §102(b) as being anticipated by Danilevich *et al.* (*Molecular Biology*, 1994, 28(1):158-166; "Danilevich"). Applicants respectfully traverse.

To reject claims as anticipated, each and every element as set forth in the claim must be found either expressly or inherently described in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). See also MPEP § 2131. Danilevich does not teach each and every element of claims 1, 2, 8, and 11.

Claim 1, the independent claim of the rejected set, is directed to a Gram-positive bacterium that has been transformed with a heterologous gene encoding pyruvate decarboxylase but that has native alcohol dehydrogenase function. Danilevich is cited as teaching "an identical Gram-positive bacterium belonging to the *Bacillus sp.* (Paper 12, page 6, lines 9-10.)

Danilevich teaches the construction of a recombinant plasmid that encodes *pdc*. The plasmid was transformed into a Gram-positive bacterium, *B. subtilis*. *B. subtilis*, however, lacks native alcohol dehydrogenase function. "The *B. subtilis* genome lacks both genes (*pdk* and *adh*)." (Page 105, second column, lines 14-15; See also page 109, first column, lines 29-30.) Thus Danilevich does not expressly or inherently teach a Gram-positive bacterium that has been transformed with a heterologous gene encoding pyruvate decarboxylase and that "has native

alcohol dehydrogenase function." Danilevich does not expressly or inherently teach each and every element of claim 1 or of dependent claims 2, 8, and 11 and does not anticipate these claims. Similarly, Danilevich does not anticipate new claims 26-31, which depend from claim 1.

Withdrawal of this rejection is respectfully requested.

Rejection of claims 1-8, 10, and 11 under 35 U.S.C. §103(a)

Claims 1-8, 10, and 11 are rejected under 35 U.S.C. §103(a) as being unpatentable over Danilevich in view of Hartley *et al.* (*Biotechnol.*, 1983, 145(1):390-396; "Hartley") and "the common knowledge in the art of molecular biology." (Paper 12, page 6, line 24.) Applicants respectfully traverse.

Each of claims 1-8, 10, and 11 recites a Gram-positive bacterium that (1) has been transformed with a heterologous gene encoding pyruvate decarboxylase and (2) has native adh function. To reject these claims as *prima facie* obvious the Patent Office must meet three criteria:

> First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

MPEP § 2143. The Patent Office has failed to meet the second criterion.

Danilevich is cited as teaching a Gram-positive bacterium with native *adh* activity that has been transformed with a plasmid that comprises a heterologous *pdc* gene. (Claim 1; Paper 12, page 6, lines 4-7.) Danilevich also is cited as teaching that the bacterium is a *Bacillus sp.* (claim 2), a heterologous *pdc* gene from *Z. mobilis* (claim 8), and transformation of the

bacterium with a plasmid comprising the heterologous gene (claim 11). (Paper 12, page 6, lines 9-11.) Hartley is cited as teaching the use of a thermophilic microorganism such as *B. stearothermophilus* for production of ethanol. (Claims 2-4; Paper 12, page 7, lines 14-15.) Hartley also is cited as teaching inactivation of the lactate dehydrogenase gene (claim 5) and a heterologous *pdc* gene from *Zymomonas sp.* or *S. cerevisiae* (claim 7). (Paper 12, page 8, lines 15-21.) The Office Action asserts:

> With the reference of Danilevich et al. which teaches the introduction of *Z. mobilis* pyruvate decarboxylase into *B. subtilis* and the reference of Hartley et al. which teaches the advantages of using a thermophilic *Bacillus* which has been genetically modified for alcohol production, it would have been obvious to one of ordinary skill in the art to transform *B. stearothermophilus* instead of *B. subtilis* as taught by Danilevich et al. and develop a strain for production of ethanol. With the common knowledge prevailing in the art of molecular biology, it would have been obvious to use methods in which the heterologous gene is introduced through a plasmid or incorporate it into the chromosome of the host bacterium. One of ordinary skill in the art would have been motivated to do so as Hartley et al. teach that use of thermophilic bacteria for production of ethanol has certain economic advantages over other fermentation methods. One of ordinary skill in the art would have a reasonable expectation of success since decarboxylase of *Z. mobilis* and the reference of Hartley et al. suggest the same and in addition provide methods to obtain lactate dehydrogenase non-producers.

Paper 12, page 8, lines 3-16.

As indicated above, Danilevich teaches that *B. subtilis* was transformed with a plasmid comprising the *pdc* gene. *B. subtilis* does not have native *adh* function. Hartley teaches manipulation of the genome of a thermophilic, Gram-positive bacterium that has a native *adh* gene (*B. stearothermophilus*) to inactivate the *lactate dehydrogenase* (*ldh*) gene. One of ordinary skill in the art, however, would not have combined these teachings to arrive at a Gram-

positive bacterium which has been transformed with a heterologous gene encoding pyruvate decarboxylase, and that has native alcohol dehydrogenase function.

Danilevich teaches that the metabolism of a bacterium having both pdc and adh gene function may be unpredictably altered: "Introduction of two actively expressed genes, *pdk* and *adh*, into *B. subtilis* may result in ethanol production or alter the cell metabolism in an unpredictable manner." (Page 109, second column, lines 33-36, emphasis added.) Thus, one of ordinary skill in the art would not have reasonably expected to be able to produce a bacterium comprising both an active, expressed heterologous pdc gene and native adh gene function.

Hartley does not remedy the deficiencies of Danilevich. Hartley teaches that "one might *try* a recombinant DNA approach to introduce and express the gene for pyruvate decarboxylase (PDC) from yeast or Zymomonas [*i.e.*, cells that have adh activity], which gives acetaldehyde + CO_2 from pyruvate (ADH then converts the acetaldehyde to ethanol)." (Page 896, line 41 to page 897, line 2, emphasis added.) But Hartley does not teach or suggest that a bacterium with both adh function and an active, expressed heterologous pdc gene could be successfully produced. In fact, if one of ordinary skill in the art looked to Danilevich for an expression vector encoding a pdc gene, she would have been taught that introduction of pdk and adh genes into a Gram-positive bacterium could alter its metabolism and would not reasonably have expected success.

In fact, in contrast to what would have been expected from the teachings of the cited art, the claimed bacteria have the unexpected properties of a significant increase in ethanol production and superior growth characteristics.

> Expression of *pdc* has resulted in a significant increase in ethanol production by the recombinant organism and has unexpectedly improved the organism's growth characteristics. Recombinant

> microorganisms, which prior to transformation with the *pdc* gene were highly unstable and difficult to culture, show significant increases in growth and survival rates both aerobically and anaerobically as well as an increase in the rate of ethanol production near to theoretical yields.

Page 4, lines 4-10.

One of ordinary skill in the art would have expected even less success at producing the bacterium of dependent claim 3. Claim 3 is directed to a <u>thermophilic</u> Gram-positive bacterium which is transformed with a heterologous gene encoding pyruvate decarboxylase that is expressed and active and which has native alcohol dehydrogenase function. Hartley teaches that a suitable vector for expression of pdc in a thermophilic bacterium was not yet available and, even if it <u>were</u> available a bacterium transfected with such a vector may not gain pdc function: "This strategy depends on developing suitable vectors for the thermophile and might fail if the enzyme were insufficiently thermostable." (Page 897, lines 2-4.)

Thus, according to Hartley, suitability of the vector is critical. Danilevich does not teach that the disclosed pdc expression vector is suitable for pdc expression in <u>thermophilic</u> bacteria. Danilevich teaches an expression vector that contains the pdc gene for use in Gram-positive bacterium. (Page 106, first column, lines 18-19.) But the vector was only tested in the Gram-positive bacterium *B. subtilis*, which is not a thermophile. Thus, the ordinary artisan would not have known whether use of the expression vector taught by Danilevich could satisfy either of the criteria set forth by Hartley: suitability for expression in thermophiles and stability of the expressed enzyme. Hartley's caveat regarding suitability of vectors, combined with Danilevich's teaching that a bacterium transfected with both adh and pdc genes may have altered metabolism and Danilevich's <u>lack</u> of teaching that the disclosed vector will express a functional pdc protein

in thermophilic bacteria, would not have provided the ordinary artisan with a reasonable expectation of success of producing the thermophilic bacterium recited in claim 3.

Withdrawal of this rejection to claims 1-8, 10, and 11 is respectfully requested.

Respectfully submitted,

Date: February 14, 2003

By: Michelle Holmes-Son

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(202) 508-9100

Amendments to the Claims

C1

1. (Currently Amended) A Gram-positive bacterium which has been transformed with a heterologous gene encoding pyruvate decarboxylase, wherein the heterologous gene expresses an active pyruvate decarboxylase, ~~or a functional equivalent thereof, but~~ and wherein the bacterium has ~~solely~~ native alcohol dehydrogenase function.

2. (Original) A Gram-positive bacterium according to claim 1 wherein the bacterium is a Bacillus sp.

C2

3. (Currently Amended) A Gram-positive bacterium according to claim 1 wherein the bacterium is a thermophile.

4. (Currently Amended) A Gram-positive bacterium according to claim 2 wherein the Bacillus is selected from B. stearothermophilus; B. calvodax; B. caldotenax, B. thermoglucosidasius, B. coagulans, B. licheniformis, B. thermodenitrificans, and B. caldolyticus.

5. (Currently Amended) A Gram-positive bacterium according to claim 1 wherein the gene encoding lactate dehydrogenase expression has been inactivated.

6. (Original) A Gram-positive bacterium according to claim 5 in which the lactate dehydrogenase gene has been inactivated by homologous recombination.

7. (Previously Amended) A Gram-positive bacterium according to claim 1 in which the heterologous gene is from Zymomonas sp or from Saccharomyces cerevisiae.

8. (Original) A Gram-positive bacterium according to claim 7 in which the heterologous gene is from Z. mobilis.

9. (Currently Amended) A Gram-positive ~~bacteria according to claim 7~~ bacterium comprising a native adh gene and which has been transformed with a ~~in which the heterologous gene is~~ pdc gene from ~~S. cerevisiae~~ *S. cerevisiae*.

C3

10. (Previously Amended) A Gram-positive bacterium according to claim 9 wherein the heterologous gene is incorporated into the chromosome of the bacterium.

11. (Previously Amended) A Gram-positive bacterium according to claim 1 in which the bacterium has been transformed with a plasmid comprising the heterologous gene.

12. (Currently Amended) A Gram-positive bacterium ~~according to claim 11,~~ comprising a native adh gene and which has been transformed with a plasmid comprising a heterologous gene encoding pyruvate decarboxylase, wherein the plasmid is pFC1.

C4

13. (Currently Amended) A Gram-positive ~~bacteria according to claim 1~~ bacterium comprising a native adh gene and which has been transformed with a heterologous gene encoding pyruvate decarboxylase wherein the heterologous gene is operatively linked to the lactate dehydrogenase promoter from Bacillus strain LN (NCIMB accession number 41038).

C5

14. (Currently Amended) Strains LN (NCIMB accession number 41038); LN-T (E31, E32); TN NCIMB accession number 41039); TN-P1; TN-P3; LN-S (J8) (NCIMB accession number 41040); LN-D (NCIMB accession number 41041); LN-D11 and ~~LN-P1~~ LN-DP1.

C6

15-25. (Canceled)

26. (New) The gram-positive bacterium of claim 9 wherein the bacterium is a thermophile.

27. (New) The gram-positive bacterium of claim 12 wherein the bacterium is a thermophile.

28. (New) The gram-positive bacterium of claim 13 wherein the bacteria is a thermophile.

29. (New) The gram-positive bacterium of claim 9 further comprising an inactivated lactate dehydrogenase gene.

30. (New) The gram-positive bacterium of claim 12 further comprising inactivated lactate dehydrogenase gene.

31. (New) The gram-positive bacterium of claim 13 further comprising inactivated lactate dehydrogenase gene.

EXHIBIT A

Merriam-Webster

Merriam-Webster's COLLEGIATE® DICTIONARY

- Home
- Help
- Word of the Day
- Word Games
- Word for the Wise
- Books and CDs
- Company Info
- Customer Service
- Network Options
- Language Zone
- The Lighter Side
- Site Map

Merriam-Webster Unabridged
Member Log-in

Atlas | Reverse Dictionary | Rhyming Dictionary
Collegiate® Dictionary | Collegiate® Thesaurus | Unabridged

One entry found for **heterologous**.

Main Entry: **het·er·ol·o·gous**
Pronunciation: -'rä-l&-g&s
Function: *adjective*
Etymology: *heter-* + *-logous* (as in *homologous*)
Date: 1893
: derived from a different species <*heterologous* DNAs> <*heterologous* transplants>
- **het·er·ol·o·gous·ly** *adverb*

Get the Top 10 Most Popular Sites for "heterologous"
For More Information on "heterologous" go to Britannica.com

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  and enjoy enhanced versions of Merriam-Webster's Collegiate® Dictionary and Thesaurus at Merriam-Webster Unabridged.
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  In fact, there's a new word waiting in your e-mail in box every morning when you subscribe to the Word of the Day.
- **Listen to Word for the Wise radio programs**
  Listen to broadcasts or read transcripts of previous Word for the Wise programs on public radio.

Pronunciation Symbols

\&\ as **a** and **u** in **abut**	\e\ as **e** in **bet**	\o\ as **aw** in **law**
\&\ as **e** in **kitten**	\E\ as **ea** in **easy**	\oi\ as **oy** in **boy**
\&r\ as **ur/er** in **further**	\g\ as **g** in **go**	\th\ as **th** in **thin**
\a\ as **a** in **ash**	\i\ as **i** in **hit**	\[th]\ as **th** in **the**
\A\ as **a** in **ace**	\I\ as **i** in **ice**	\ü\ as **oo** in **loot**
\ä\ as in **mop**	\j\ as **j** in **job**	\u\ as **oo** in **foot**
\au\ as **u** in **ut**	\[ng]\ as **ng** in **sing**	\y\ as **y** in **yet**
\ch\ as **ch** in **chin**	\O\ as **o** in **go**	\zh\ as **si** in **vision**

EXHIBIT B

PubMed Nucleotide Protein Genome Structure PopSet Taxonomy

Search Nucleotide for Go Clear

Limits Preview/Index History Clipboard Details

Display default Save Text Add to Clipboard Get Subsequence

□ **1:** X87929. K.lactis PDC gene...[gi:3688421] Links

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VERSION     X87929.1  GI:3688421
KEYWORDS    consensus sequence; CYC1 gene; PDC gene; promoter region; pyruvate
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  ORGANISM  Kluyveromyces lactis
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  AUTHORS   Ramil,E., Freire-Picos,M.A. and Cerdan,M.E.
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            KlCYC1
  JOURNAL   Eur. J. Biochem. 256 (1), 67-74 (1998)
  MEDLINE   98417429
REFERENCE   2
  AUTHORS   Cerdan,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-JUN-1995) E. Cerdan, Univ. de la Coruna, Departamento
            de Biologia Celular y Mol., Facultad de Ciencias, Campus de La
            Zapateira s/n, E- 15071 La Coruna, SPAIN
REFERENCE   3  (bases 1 to 3008)
  AUTHORS   Cerdan,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-NOV-1995) M.E. Cerdan, Univ. de la Coruna,
            Departamento de Biologia Celular y Mol., Facultad de Ciencias,
            Campus de La Zapateira s/n, E- 15071 La Coruna, SPAIN
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PubMed Nucleotide Protein Genome Structure PopSet Taxonomy

Search Nucleotide for Go Clear

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1: X81854. N.tabacum mRNA fo...[gi:551260] Links

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LOCUS       NTMRNAPD1               1255 bp    mRNA    linear   PLN 06-NOV-1995
DEFINITION  N.tabacum mRNA for pyruvate decarboxylase 1.
ACCESSION   X81854
VERSION     X81854.1  GI:551260
KEYWORDS    pyruvate decarboxylase; tobPDC1.
SOURCE      common tobacco.
  ORGANISM  Nicotiana tabacum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;
            Asteridae; euasterids I; Solanales; Solanaceae; Nicotiana.
REFERENCE   1  (bases 1 to 1255)
  AUTHORS   Bucher,M., Brander,K.A., Sbicego,S., Mandel,T. and Kuhlemeier,C.
  TITLE     Aerobic fermentation in tobacco pollen
  JOURNAL   Plant Mol. Biol. 28 (4), 739-750 (1995)
  MEDLINE   95375236
REFERENCE   2  (bases 1 to 1255)
  AUTHORS   Mandel,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-SEP-1994) T. Mandel, Institute of Plant Physiology,
            Altenbergrain 21, 3013 Bern, FRG
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EXHIBIT D

☐ **1:** U71122. Arabidopsis pyruv...[gi:1616786] Links

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LOCUS       ATU71122                4069 bp    DNA     linear   PLN 08-OCT-1996
DEFINITION  Arabidopsis pyruvate decarboxylase-2 (Pdc2) gene, complete cds.
ACCESSION   U71122
VERSION     U71122.1  GI:1616786
KEYWORDS    .
SOURCE      Arabidopsis thaliana.
  ORGANISM  Arabidopsis thaliana
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;
            Rosidae; eurosids II; Brassicales; Brassicaceae; Arabidopsis.
REFERENCE   1  (bases 1 to 4069)
  AUTHORS   Dolferus,R., Peacock,W.J. and Dennis,E.S.
  TITLE     Two pyruvate decarboxylase genes from Arabidopsis
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 4069)
  AUTHORS   Dolferus,R., Peacock,W.J. and Dennis,E.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-SEP-1996) Plant Industry, C.S.I.R.O., G.P.O. Box
            1600, Canberra, Act. 2601, Australia
FEATURES             Location/Qualifiers
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Revised: July 5, 2002.

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1: U65927. Neurospora crassa...[gi:1655908] Links

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LOCUS       NCU65927                3741 bp    DNA     linear   PLN 01-NOV-1996
DEFINITION  Neurospora crassa pyruvate decarboxylase (cfp) gene, promoter
            region, and complete cds.
ACCESSION   U65927
VERSION     U65927.1  GI:1655908
KEYWORDS    .
SOURCE      Neurospora crassa.
  ORGANISM  Neurospora crassa
            Eukaryota; Fungi; Ascomycota; Pezizomycotina; Sordariomycetes;
            Sordariales; Sordariaceae; Neurospora.
REFERENCE   1  (bases 1 to 1699)
  AUTHORS   Temporini,E., Alvarez,M., Mautino,M., Kawaguchi,T., Kinghorn,J. and
            Rosa,A.
  TITLE     Cloning, sequence and functional analysis of the Neurospora crassa
            pyruvate decarboxylase gene promoter
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 3741)
  AUTHORS   Temporini,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-AUG-1996) Esteban Temporini, Facultad de Ciencias
            Quimicas-Univ. Nac. Cordoba, Departamento de Quimica Biologica,
            Ciudad Universitaria s/n, Cordoba 5016, Argentina
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PubMed Nucleotide Protein Genome Structure PopSet Taxonomy

Search Nucleotide for Go Clear

Limits Preview/Index History Clipboard Details

Display default Save Text Add to Clipboard Get Subsequence

1: X92743. O.sativa mRNA for...[gi:1054820] Links

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LOCUS       OSPDECOOH                856 bp    mRNA    linear   PLN 07-AUG-1997
DEFINITION  O.sativa mRNA for pyruvate decarboxylase.
ACCESSION   X92743
VERSION     X92743.1  GI:1054820
KEYWORDS    pyruvate decarboxylase.
SOURCE      Oryza sativa.
  ORGANISM  Oryza sativa
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae;
            Ehrhartoideae; Oryzeae; Oryza.
REFERENCE   1
  AUTHORS   Rivoal,J., Thind,S., Pradet,A. and Ricard,B.
  TITLE     Differential induction of pyruvate decarboxylase subunits and
            transcripts in anoxic rice seedlings
  JOURNAL   Plant Physiol. 114 (3), 1021-1029 (1997)
  MEDLINE   97377132
REFERENCE   2  (bases 1 to 856)
  AUTHORS   Ricard,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-NOV-1995) B.C. Ricard, Institut National de la
            Recherche Agron., Station de Physiologie vegetale, B.P.81, F-33883
            Villenave d'Ornon Cedex, FRANCE
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PubMed Nucleotide Protein Genome Structure PopSet Taxonomy

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Display default Save Text Add to Clipboard Get Subsequence

1: X59546. Z.mays Pdc mRNA f...[gi:22394] Links

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DEFINITION  Z.mays Pdc mRNA for pyruvate decarboxylase.
ACCESSION   X59546
VERSION     X59546.1  GI:22394
KEYWORDS    anaerobic stress response; glycolytic enzyme; PDC gene; pyruvate
            decarboxylase.
SOURCE      Zea mays.
  ORGANISM  Zea mays
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACC
            clade; Panicoideae; Andropogoneae; Zea.
REFERENCE   1  (bases 1 to 3791)
  AUTHORS   Kelley,P.M., Godfrey,K., Lal,S.K. and Alleman,M.
  TITLE     Characterization of the maize pyruvate decarboxylase gene
  JOURNAL   Plant Mol. Biol. 17 (6), 1259-1261 (1991)
  MEDLINE   92032792
REFERENCE   2  (bases 1 to 3791)
  AUTHORS   Kelley,P.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-MAY-1991) P.M. Kelley, School of Biological Sciences,
            University of Nebraska-Lincoln, 245 Manter Hall, Lincoln, Nebraska,
            68588-0118, USA
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EXHIBIT H

1: U75311. Pichia stipitis p...[gi:2734882] Links

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DEFINITION  Pichia stipitis pyruvate decarboxylase 2 (PsPDC2) gene, complete
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ACCESSION   U75311
VERSION     U75311.1  GI:2734882
KEYWORDS    .
SOURCE      Pichia stipitis.
  ORGANISM  Pichia stipitis
            Eukaryota; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes;
            Saccharomycetales; Saccharomycetaceae; Pichia.
REFERENCE   1  (bases 1 to 2305)
  AUTHORS   Lu,P., Davis,B.P. and Jeffries,T.W.
  TITLE     Cloning and characterization of two pyruvate decarboxylase genes
            from Pichia stipitis CBS 6054
  JOURNAL   Appl. Environ. Microbiol. 64 (1), 94-97 (1998)
  MEDLINE   98096791
   PUBMED   9435065
REFERENCE   2  (bases 1 to 2305)
  AUTHORS   Lu,P., Davis,B.P. and Jeffries,T.W.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-OCT-1996) Institute for Microbial and Biochemical
            Technology, USDA, FS, Forest Products Laboratory, One Gifford
            Pinchot Drive, Madison, WI 53705, USA
FEATURES             Location/Qualifiers
     source          1..2305
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Revised: July 5, 2002.

Oct 3 2002 17:48:23

1: AF098293. Aspergillus oryza...[gi:4323052] Links

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ACCESSION   AF098293
VERSION     AF098293.1  GI:4323052
KEYWORDS    .
SOURCE      Aspergillus oryzae.
  ORGANISM  Aspergillus oryzae
            Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes;
            Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Aspergillus.
REFERENCE   1  (bases 1 to 2525)
  AUTHORS   Lee,D.W., Koh,J.S., Kim,J.H. and Chae,K.-S.
  TITLE     Cloning and nucleotide sequence of one of the most highly expressed
            genes, a pdcA homologue of Aspergillus nidulans, in Aspergillus
            oryzae
  JOURNAL   Biotechnol. Lett. 21, 139-142 (1999)
REFERENCE   2  (bases 1 to 2525)
  AUTHORS   Lee,D.W., Koh,J.S., Kim,J.H. and Chae,K.-S.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-OCT-1998) Faculty of Biological Sciences, Chonbuk
            National University, Dukjin-dong 1-ga, 664-14, Chonju, Chonbuk
            561-756, Republic of Korea
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Revised: July 5, 2002.

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NCBI | NLM | NIH

Oct 3 2002 17:48:23

1: AI728334. BNLGHi10479 Six-d...[gi:5047186]

IDENTIFIERS

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dbEST Id:       2637583
EST name:       BNLGHi10479
GenBank Acc:    AI728334
GenBank gi:     5047186
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CLONE INFO

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Clone Id:       (5')
DNA type:       cDNA
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PRIMERS

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Sequencing:     T3 Primer
PolyA Tail:     Unknown
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SEQUENCE

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Entry Created:  Jun 11 1999
Last Updated:   Jun 11 1999

PUTATIVE ID     Assigned by submitter
                PYRUVATE DECARBOXYLASE ISOZYME 1 (PDC)
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LIBRARY

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Lib Name:       Six-day Cotton fiber
Organism:       Gossypium hirsutum
Cultivar:       Acala Maxxa
Tissue type:    immature fiber
Develop. stage: Six days post anthesis
Lab host:       XL1-Blue
Vector:         pBluescript II KS+
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SUBMITTER

```
Name:           Ben Burr
Lab:            Biology Department
Institution:    Brookhaven National Laboratory
```

Address: Upton, NY 11973, USA
Tel: 516-344-3396
Fax: 516-344-3407
E-mail: burr@bnlux1.bnl.gov

CITATIONS

Title: ESTs from developing cotton fiber
Authors: Blewitt,M., Matz,E.C., Davy,D.F., Burr,B.
Year: 1999
Status: Unpublished

MAP DATA

Revised: July 5, 2002.

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EXHIBIT K

PubMed Nucleotide Protein Genome Structure PopSet Taxonomy

Search Nucleotide for Go Clear

Limits Preview/Index History Clipboard Details

Display default Save Text Add to Clipboard Get Subsequence

1: Z54096. S.pombe chromosom...[gi:984221] Links

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LOCUS       SPAC13A11              12168 bp    DNA     linear   PLN 18-OCT-1999
DEFINITION  S.pombe chromosome I cosmid c13A11.
ACCESSION   Z54096
VERSION     Z54096.1  GI:984221
KEYWORDS    cyp51; cytochrome P450; cytosol aminopeptidase; Fes/CIP4 homology
            domain; pyruvate decarboxylase; Rho GTPase protein; Thiamine
            pyrophosphate enzymes; ubiquitin carboxy-terminal hydrolase.
SOURCE      fission yeast.
  ORGANISM  Schizosaccharomyces pombe
            Eukaryota; Fungi; Ascomycota; Schizosaccharomycetes;
            Schizosaccharomycetales; Schizosaccharomycetaceae;
            Schizosaccharomyces.
REFERENCE   1  (bases 1 to 12168)
  AUTHORS   Hunt,S., Devlin,K., Churcher,C.M., Barrell,B.G., Rajandream,M.A.
            and Walsh,S.V.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-SEP-1995) Schizosaccharomyces pombe chromosome I
            sequencing project, Sanger Centre, Hinxton Hall, Hinxton, Cambridge
            CB10 1RQ E-mail: barrell@sanger.ac.uk
COMMENT     Notes:
            Details of yeast sequencing at the Sanger Centre are available on
            the World Wide Web.
            (URL, http://www.sanger.ac.uk/Projects/S_pombe)
            Protein coding regions (CDS) have been predicted with the help of
            computer analysis using the Genefinder program in PomBase (an ACEDB
            database) with additional predictions for the branch-acceptor sites
            supplied by the program Sp3splice. CAUTION: It is possible that for
            any individual CDS we may have underestimated or overestimated the
            number of introns/exons or we may not have chosen the correct
            splice donor/acceptor sites.
            CDS are numbered using the following system eg SPAC5H10.01c. SP (S.
            pombe), A (chromosome 1), c5H10 (cosmid name), .01 (first CDS), c
            (complementary strand).
            The more significant matches with motifs in the PROSITE database
            are also included but some of these may be fortuitous.
            The length in codons is given for each CDS.
            IMPORTANT: This sequence MAY NOT be the entire insert of the
            sequenced clone.  It may be shorter because we only sequence
            overlapping sections once, or longer, because we arrange for a
            small overlap between neighbouring submissions.
            Cosmid c13A11 overlapped at the 5' end by comsmid c2F7 and at the
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FEATURES             Location/Qualifiers
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 9721 gcagttcact atgtctcttc tcagtataag accaaggctg tgattgaagc cagcacatta
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 9901 ccatttcacg aggcttacct caagcaactt acaagttctt caaatgctga tttgtgcaat
 9961 gttagtcgtg ccggtggtgg ttgttgtaca gcggctgcct ttattaaatg cttcttagcc
10021 cagaaagatt tgtcttttgc tcatttagac attgccggag ttatggataa gcagcttaat
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10201 ctgttgatta caccaatctc atttttcaat gtccttgtta agtgtatgtc aatgatatgt
10261 agctatcatg attttattct ttttaatact gttttccaca accgttcata acggttgtaa
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10381 gaagtctcat gttgttaaat ttcactttcg aagtagcgaa tttttttttt ctattttgaa
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11461 aaagaataat ccacattaac tcaatgtatc attgatgacg gaatgttgct tatatagcca
11521 gagtaacccт gctctgaagc atctaaggtt atatcttttt aacttttagc tagcaaactc
11581 acttgttcct ttaagttcag aaagtatctg ctggttattc aacagataat tatcttgcga
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11701 gcattatcaa acatgagtgg ggatatttta gtcggtgaat atctattcaa aaggcttgaa
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12001 ccagtagttc atattgttgg aatgccttcc acaaaggtgc aagatactgg agctttgctt
12061 catcatactt taggagatgg agactttcgc actttcatgg atatgtttaa gaaagtttct
12121 gcctacagta taatgatcga taacggaaac gatgcagctg aaaagatc
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Characterization of *Bacillus stearothermophilus* Plasmid pAB124 and Construction of Deletion Variants

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A restriction endonuclease cleavage map of the tetracycline resistance plasmid pAB124, originally isolated from *Bacillus stearothermophilus*, was constructed using ten enzymes. Tetracycline resistance was associated with a 1·95 megadalton (Md) region of pAB124 lying between two *Eco*RI sites, and this region was circularized to produce a viable tetracycline resistance plasmid (pAB224), with two *Eco*RI fragments of pAB124 deleted amounting to 0·95 Md. A second plasmid (pAB524) with one *Eco*RI fragment (0·6 Md) of pAB124 deleted was also constructed. Restriction endonuclease cleavage maps of pAB224 and pAB524 were constructed.

INTRODUCTION

Several plasmids have been isolated from Gram-positive spore-forming bacilli, e.g. *Bacillus pumilus* (Lovett & Bramucci, 1975), *B. megaterium* (Carlton & Helinski, 1969) and *B. subtilis* (Tanaka *et al.*, 1977; Le Hegarat & Anagnostopoulos, 1977), although none of these has been shown to be associated with any phenotypic trait. More recently, a plasmid from *B. cereus* coding for tetracycline resistance and one from *B. subtilis* producing a bacteriocin have been isolated (Bernhard *et al.*, 1978).

The presence of a plasmid in a bacteriocin (thermocin)-producing strain of *B. stearothermophilus* has recently been reported (Sharp *et al.*, 1979), and we have described the isolation of four plasmids from antibiotic-resistant thermophilic bacilli (Bingham *et al.*, 1979), one of which (pAB124) was shown to confer tetracycline resistance on *B. subtilis*. This paper describes the characterization of pAB124 and the construction of deletion variants of this plasmid.

METHODS

Bacterial strains. Thermophilic *Bacillus* strain TB124, isolated as previously described (Bingham *et al.*, 1979), was classified as *Bacillus stearothermophilus* using standard identification procedures (Cowan & Steel, 1974). *Bacillus subtilis* harbouring plasmid pAB124 was prepared by direct plasmid transformation. *Bacillus subtilis* IG20 (*hsr hsm trp*) was kindly provided by A. Docherty, Department of Bacteriology, Bristol University.

Culture media. For plasmid isolation all strains were grown on TYS medium (Bingham *et al.*, 1979) supplemented with tetracycline at 25 $\mu g\ ml^{-1}$. The following minimal medium (SMS) was used for *B. subtilis* ($g\ l^{-1}$): $(NH_4)_2SO_4$, 2; K_2HPO_4, 14; KH_2PO_4, 6; sodium citrate, 1; $MgSO_4.7H_2O$, 0·2; glucose, 5.

Isolation of plasmid DNA. This was carried out with 400 ml cultures using the procedure previously described (Bingham *et al.*, 1979). Plasmid pAB124 was originally isolated from *B. stearothermophilus* TB124 and transformed into *B. subtilis* (Bingham *et al.*, 1979). All experiments described in this paper used pAB124 isolated from *B. subtilis*. Plasmid pUB1654 was a gift from A. Docherty.

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Small-scale plasmid preparation. This was used for extracting recombinant DNA from large numbers of clones. Cultures (15 ml) in TYS medium containing the selective antibiotic(s) were grown overnight at 37 °C without shaking. Cells were harvested at 10000 *g*, washed once in 5 ml TES buffer (30 mM-Tris/HCl pH 8·0, 5 mM-Na_2EDTA, 50 mM-NaCl) and resuspended in 100 μl TES. EDTA (25 μl, 0·25 M) and lysozyme (25 μl, 15 mg ml^{-1}) were added and the suspension was shaken gently on ice for 10 min. The suspension was then placed in a 37 °C water bath for 5 min and 25 μl sodium dodecyl sulphate (10 %, w/v) was added, to bring about lysis, followed by 50 μl NaCl (5 M); this mixture was left at 0 °C for 2·5 to 3 h. A cleared lysate was obtained by centrifugation at 45000 *g* for 45 min, and this was then extracted twice with chloroform/3-methylbutan-1-ol (24:1, v/v), once with phenol (freshly distilled over N_2) equilibrated in TES buffer and three times with diethyl ether. The plasmid DNA obtained was suitable for restriction endonuclease analysis.

Plasmid transformation. Bacillus subtilis IG20 was grown overnight at 37 °C in 200 ml SMS medium supplemented with 0·5 ml tryptophan (2 mg ml^{-1}) and then diluted with an equal volume of similar medium (prewarmed). The absorbance (540 nm) was monitored at 30 min intervals, and at a value equivalent to maximum competence (30 to 60 min after cessation of exponential growth) 1 ml samples of the culture were added to plasmid DNA (0·5 to 5 μg) in 50 μl polyethylene glycol 1000 (50 %, w/v) and incubated for 1 h at 37 °C with vigorous aeration. Prewarmed TYS medium (5 ml) was then added and incubation was continued for a further hour prior to plating on selective media. Selective concentrations of antibiotics used were 25 μg ml^{-1} for tetracycline and neomycin and 75 μg ml^{-1} for streptomycin. It was not necessary to induce competence by dilution of the culture into a starvation medium (Stacey, 1968).

*Restriction endonucleases. Eco*RI and *Cau*II were purified by procedures developed in this laboratory and digestions were carried out in buffers previously described (Bingham *et al.*, 1979). *Bst*EII, *Hae*III, *Hha*I, *Hin*dII, *Hpa*I, *Hpa*II, *Tha*I, *Xba*I and T4-DNA ligase were purchased from Uniscience Ltd, Cambridge, and digestions carried out in the buffers recommended by the manufacturer. Reaction mixtures contained 0·5 to 1 μg plasmid DNA in a final volume of 20 to 25 μl, and reactions were terminated by adding Na_2EDTA to a final concentration of 10 mM (*Cau*II, *Bst*EII, *Tha*I) or heating at 66 °C for 10 min. For double digestions involving enzymes with different buffer requirements, plasmid DNA was first digested with the enzyme requiring the buffer of lower ionic strength, and then the buffer was adjusted with 10× concentrated components prior to the addition of the second enzyme.

Agarose gels (0·8 %, w/v) were used as previously described (Bingham *et al.*, 1979), with an *Eco*RI plus *Hin*dIII λ C*I*857 digest providing fragments of standard sizes: 13·4, 3·35, 3·2, 2·8, 2·32, 1·39, 1·27, 1·05, 0·89, 0·59, 0·47 and 0·31 megadaltons (Md) (Murray & Murray, 1975). The sizes of unknown fragments were determined graphically from a 10× enlargement of a photograph (35 mm) and were taken as an average of three estimations.

Cloning of pAB124 *Eco*RI *fragments.* Plasmids pAB124 (1 μg) and pUB1654 (1 μg) were digested to completion with *Eco*RI and then the enzyme was denatured by heating at 66 °C for 10 min. The fragments were precipitated by adding 3 M-sodium acetate to a final concentration of 0·3 M and 2 vol. cold (−20 °C) ethanol. After 10 min in a solid CO_2/ethanol bath the precipitate was collected by centrifugation. After removal of all the ethanol the precipitate was resuspended in T4 ligase buffer (66 mM-Tris/HCl pH 7·6, 6·6 mM-$MgCl_2$, 10 mM-dithiothreitol, 0·4 mM-ATP) and incubated overnight at 4 °C with 0·05 units T4-DNA ligase.

RESULTS

Restriction endonuclease site mapping of pAB124

The fragment sizes obtained by digestion of pAB124 DNA (isolated from *B. subtilis*) with *Bst*EII, *Cau*II, *Eco*RI, *Hae*III, *Hin*dII, *Hha*I, *Hpa*I, *Hpa*II, *Tha*I and *Xba*I are given in Table 1. These sizes were determined graphically using a lambda DNA digested with *Hin*dIII plus *Eco*RI as standards subjected to electrophoresis on the same gels as the unknown fragments. The average value for the total size of pAB124 was close to 2·9 Md. A series of double digestion experiments was carried out in order to construct a cleavage map of pAB124 (Table 2).

The positions of the four single sites (*Bst*EII, *Cau*II, *Hpa*I, *Xba*I) relative to each other were determined taking *Cau*II as the reference point. An *Hpa*I plus *Cau*II digest and a *Bst*EII plus *Cau*II digest both gave a small fragment of 0·35 Md; thus the *Bst*EII and *Hpa*I sites are the same distance from *Cau*II site either on the same side, i.e. very close together, or on opposite sides 0·7 Md apart. The latter position was confirmed by a *Bst*EII plus *Hpa*I double digestion. The *Cau*II plus *Xba*I digestion generated fragments of 1·85 and 1·05 Md. It was assumed that the *Xba*I site was 1·85 Md to the right of *Cau*II and the *Hpa*I

Table 1. *Fragments produced by restriction endonuclease digestion of* pAB124

Digestions and agarose-gel electrophoresis were carried out as described in Methods using plasmid DNA isolated from *B. subtilis.*

Enzyme	Fragment size (Md) A	B	C	Sum of fragment sizes (Md)
*Bst*EII	2·90			2·90
*Cau*II	2·90			2·90
*Hpa*I	2·90			2·90
*Xba*I	2·90			2·90
*Hha*I	2·23	0·70		2·93
*Hind*II	2·30	0·62		2·92
*Hae*III	1·50	1·18	0·25	2·93
*Hpa*II	1·80	0·70	0·42	2·92
*Tha*I	1·84	0·65	0·40	2·89
*Eco*RI	1·95	0·61	0·33	2·89

Table 2. *Fragments produced by digestion of* pAB124 *with two restriction endonucleases*

Double digestions and agarose-gel electrophoresis were carried out as described in Methods using plasmid DNA isolated from *B. subtilis.*

Enzyme pair	Fragment size (Md) A	B	C	D	Sum of fragment sizes (Md)
*Bst*EII + *Cau*II	2·55	0·35			2·90
*Bst*EII + *Hpa*I	2·23	0·70			2·93
*Bst*EII + *Xba*I	2·20	0·70			2·90
*Cau*II + *Hpa*I	2·57	0·35			2·92
*Cau*II + *Xba*I	1·85	1·05			2·90
*Hpa*I + *Xba*I	1·48	1·40			2·88
*Hha*I + *Cau*II	2·20	0·44	0·25		2·89
*Hha*I + *Xba*I	1·59	0·71	0·60		2·90
*Hha*I + *Hpa*I	2·10	0·70	0·10		2·90
*Hind*II + *Cau*II	2·30	0·33	0·28		2·91
*Hind*II + *Xba*I	1·50	0·78	0·62		2·90
*Hind*II + *Hpa*I	2·30	0·61	—		2·91
*Hae*III + *Cau*II	1·24	1·18	0·25	0·24	2·91
*Hae*III + *Bst*EII	1·18	0·89	0·59	0·25	2·91
*Hae*III + *Xba*I	1·30	1·18	0·25	0·18	2·91
*Hae*III + *Hpa*I	1·51	1·19	0·16	0·10	2·96
*Hpa*II + *Cau*II	1·80	0·70	0·42	—	2·92
*Hpa*II + *Bst*EII	1·80	0·42	0·35	0·32	2·89
*Hpa*II + *Xba*I	1·80	0·70	0·38	(0·05)†	2·93
*Hpa*II + *Hpa*I	1·45	0·72	0·42	0·35	2·94
*Tha*I + *Cau*II	1·80	0·42	0·40	0·23	2·85
*Tha*I + *Bst*EII	1·83	0·62	0·40	(0·05)†	2·90
*Tha*I + *Xba*I	1·60	0·65	0·40	0·26	2·91
*Tha*I + *Hpa*I	1·75	0·65	0·40	0·10	2·90
*Eco*RI + *Cau*II	1·55	0·60	0·40	0·33	2·88
*Eco*RI + *Bst*EII	1·90	0·62	0·33	(0·05)†	2·90
*Eco*RI + *Xba*I	1·95	0·33*	0·29	—	2·88
*Eco*RI + *Hpa*I	1·20	0·75	0·60	0·32	2·87

* This fragment was overabundant and assumed to be a 'double'.
† Fragment not detected, but its presence can be predicted.

and *Bst*EII sites were positioned using this orientation. If *Hpa*I lies 0·35 Md to the right of the *Cau*II site a double digestion with *Hpa*I plus *Xba*I would generate 1·48 and 1·40 Md fragments, whereas if it lies 0·35 Md to the left, 0·8 and 2·1 Md fragments would be generated. Since fragments of the former sizes were obtained, the *Hpa*I site must lie to the right of the

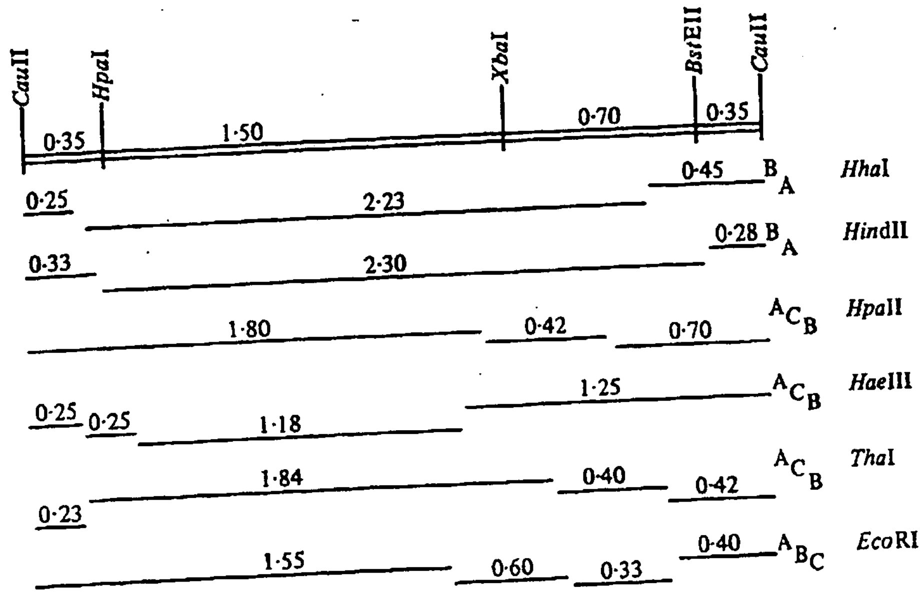


Fig. 1. Relative positions of fragments produced by digestion of pAB124 with several restriction endonucleases.

*Cau*II site with the *Bst*EII site 0·35 Md to the left. The positions of the four sites (Fig. 1) were consistent with the results of all the double digestions carried out.

Using these four single sites as reference points the fragments generated by *Hha*I, *Hin*dII, *Hpa*II, *Hae*III, *Tha*I and *Eco*RI (Table 1) were ordered using the established techniques of double-digestion mapping. For example, the *Eco*RI fragments were ordered easily as only one position of *Eco*RI fragment A was consistent with it containing the *Bst*EII, *Cau*II and *Hpa*I sites but not the *Xba*I site which is present in *Eco*RI fragment B. A *Cau*II plus *Eco*RI digestion generated 1·55 amd 0·4 Md fragments from *Eco*RI A; the 1·55 Md fragment could only lie to the right of the *Cau*II site with the 0·4 Md fragment to the left (Fig. 1). The *Eco*RI B fragment must lie to the right of *Eco*RI A over the *Xba*I site in the position shown (Fig. 1) since an *Eco*RI plus *Xba*I digest yielded 0·33 and 0·29 Md fragments from *Eco*RI B and the distance from *Eco*RI A to the *Xba*I site is 0·35 Md. The remaining fragment, *Eco*RI C, therefore lies to the right of *Eco*RI B.

The fragments generated by digestion of pAB124 with *Hha*I, *Hin*dII, *Hae*III, *Hpa*II and *Tha*I were ordered in a similar manner and their relative positions are shown in Fig. 1.

*Cloning of Eco*RI *fragments of* pAB124 *carrying the tetracycline resistance gene*

Staphylococcal plasmid pUB1654 conferring streptomycin and neomycin resistance was used. This has a single *Eco*RI site lying within the streptomycin resistance gene (A. Docherty, personal communication). It would therefore be expected that insertion of pAB124 fragments at this site would inactivate streptomycin resistance. Transformation of the ligated fragments generated about 35% of clones that were Nm^r Str^s Tc^r. DNA was extracted from several of these clones and was analysed with *Eco*RI (Table 3). The minimum requirement for tetracycline resistance was shown to be the *Eco*RI fragment A (1·95 Md) of pAB124 present alone in 50% of the recombinant molecules examined.

*Construction of small tetracycline resistance plasmids from cloned Eco*RI *fragments of* pAB124

Plasmid DNA from each type of clone, representing all combinations of pAB124 *Eco*RI fragments (Table 3), was digested to completion with *Eco*RI and then ligated with T4-DNA ligase. The DNA was then transformed into *B. subtilis* IG20 and Tc^r Nm^s Str^s clones were identified. Two types of plasmid were isolated from the clones examined (Table 4): pAB224

Table 3. *Restriction endonuclease analysis of recombinant plasmids of* pAB124 *and* pUB1654

Plasmid DNA was extracted from clones of Nm^r Str^s Tc^r phenotype and digested with *Eco*RI as described in Methods.

Clones identified		*Eco*RI digestion		
No.	Plasmid size (Md)	Fragment size (Md)	pAB124 fragment(s) inserted	Type of recombinant
10	6·0	4·0*	—	I
		1·95	A	
3	6·55	4·0*	—	II
		1·95	A	
		0·60	B	
2	6·30	4·0*	—	III
		1·95	A	
		0·32	C	
4	6·90	4·0*	—	IV
		1·95	A	
		0·60	B	
		0·32	C	

* This fragment corresponds to pUB1654.

Table 4. *Eco*RI *analysis of tetracycline resistance plasmids derived from* pAB124:pUB1654 *recombinant molecules*

Plasmid DNA was extracted from Tc^r Nm^s Str^s clones and digested with *Eco*RI as described in Methods.

DNA source*		Tetracycline resistance plasmids			
Type of recombinant	pAB124 *Eco*RI fragment(s) present	No. of clones†	Size (Md)	*Eco*RI fragments (Md)	pAB124 *Eco*RI fragment(s) present
I	A	10	1·95	1·95	A (pAB224)
II	A+B	10	1·95	1·95	A (pAB224)
III	A+C	7	1·95	1·95	A (pAB224)
		3	2·3	1·95, 0·32	A+C (pAB524)
IV	A+B+C	8	1·95	1·95	A (pAB224)
		2	2·3	1·95, 0·32	A+C (pAB524)

* Recombinant plasmids of pAB124 and pUB1654 (see Table 3).
† Ten clones from each transformation were examined.

which was the recircularized *Eco*RI fragment A of pAB124 (1·95 Md) and pAB524 containing *Eco*RI fragments A plus C (2·3 Md). No plasmids containing *Eco*RI fragments A plus B or reconstructed pAB124 were detected.

Plasmid pAB224 was shown to contain seven single sites (*Eco*RI, *Bst*EII, *Cau*II, *Hpa*II, *Hha*I, *Tha*I and *Hpa*I). The position of each site was determined by double digestions and was shown to be consistent with pAB224 being the recircularized *Eco*RI fragment A of pAB124 (Fig. 2). Therefore *Eco*RI A contains the tetracycline resistance genes and all the known essential functions of pAB124.

An *Eco*RI digest of pAB524 generated fragments of 1·95 Md and 0·32 Md. The larger fragment contained *Bst*EII, *Cau*II and *Hpa*I sites and double digestions confirmed that it was the *Eco*RI fragment A of pAB124; the smaller fragment contained *Hha*I and *Hpa*II sites and was assumed to be the *Eco*RI fragment C of pAB124. Two orientations of *Eco*RI C within pAB524 were possible and each would give different sized fragments when digested with *Hpa*II or *Hha*I. The observed sizes of the fragments obtained from five different examples of pAB524 indicated that the order of *Eco*RI fragments A and C was the same as that observed in pAB124 (Fig. 2).

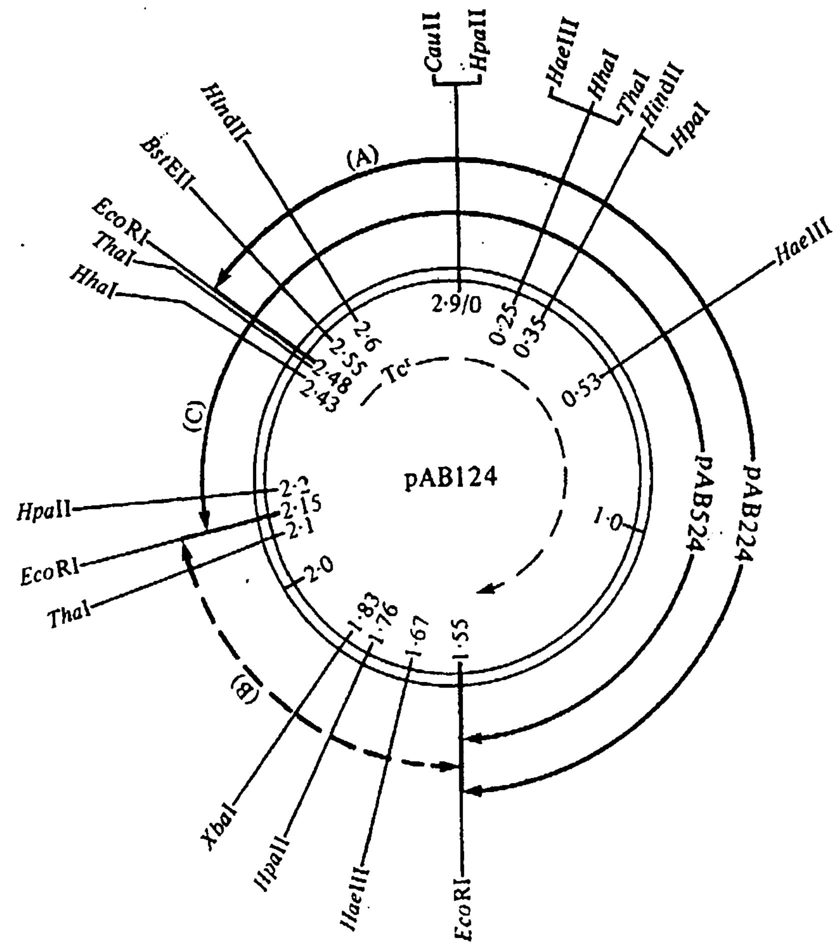
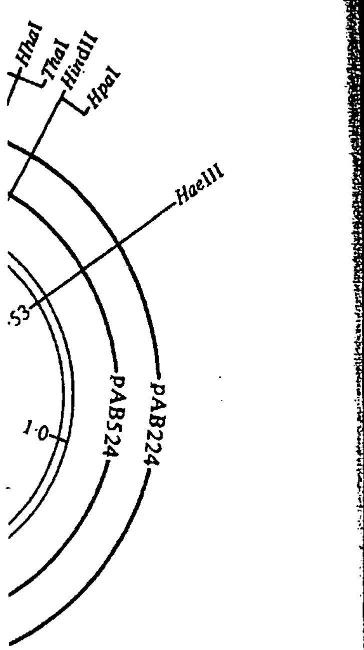


Fig. 2. Restriction endonuclease cleavage map of pAB124, pAB224 and pAB524.

DISCUSSION

Classification of the thermophilic bacilli harbouring pAB124 by standard procedures indicated that the strains were quite closely related to *B. stearothermophilus* but not identical to the neotype strain NCA 1503. The aim of this research is to develop a vector for cloning in *B. stearothermophilus* and pAB124 could provide the basis for such a vector. The expression of pAB124 in *B. subtilis* will also allow the use of this plasmid as a vector for gene cloning in this organism, and it is important to note that the plasmid is stable with no apparent alteration upon uptake and replication in *B. subtilis*, as determined by restriction endonuclease analysis. The transformation frequencies, however, are lower with plasmid DNA isolated from *B. stearothermophilus* than with that from *B. subtilis*; this may in part be due to the formation of concatemers in *B. subtilis* that transform at a higher frequency than the monomeric species (Canosi *et al.*, 1978). The most convenient restriction site for cloning in pAB124 is the single *Xba*I, since this enzyme produces a cohesive terminus and lies within the region of pAB124 not essential for tetracycline resistance or plasmid replication (Fig. 2). We have successfully inserted a staphylococcal neomycin resistance plasmid (pUB110) at the *Xba*I site without inactivating tetracycline resistance. Of the remaining single restriction sites in pAB124, it is not known whether *Bst*EII and *Cau*II produce a cohesive terminus and *Hpa*I is known to produce base-paired termini, thus making the use of these sites for cloning more difficult. In addition, these latter sites lie within the region of pAB124 that appears to be associated with tetracycline resistance.

The development of the staphylococcal vector pUB1654 (Nm^r Str^r; A. Docherty, personal communication) allowed us to examine which region of pAB124 contained the



124, pAB224 and pAB524.

pAB124 by standard procedures
:earothermophilus but not identical
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ıvenient restriction site for cloning
a cohesive terminus and lies within
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ıycin resistance plasmid (pUB110)
ce. Of the remaining single restric-
*Cau*II produce a cohesive terminus
s making the use of these sites for
within the region of pAB124 that

654 (Nm^r Str^r; A. Docherty, per-
region of pAB124 contained the

tetracycline resistance genes. Only the large *Eco*RI fragment (A) was essential for the expression of the Tc^r phenotype and the survival of the plasmid as a replicon. The circularized *Eco*RI fragment A (pAB224) contained one site for each of seven restriction endonucleases (Fig. 2); three of these enzymes produce a cohesive terminus (*Eco*RI, *Hpa*II and *Hha*I), and therefore this plasmid is of potential use as a small (1·95 Md) vector for gene cloning. We have attempted to insert at the *Eco*RI site the *B. licheniformis* β-lactamase that is cloned between two *Eco*RI sites in a lambda vector (W.J. Brammar, personal communication) and also the β-lactamase of the staphylococcal plasmid pI258 (Novick *et al.*, 1979). In both cases we could not isolate recombinant molecules containing a β-lactamase gene. We are therefore continuing to examine the ability of pAB224 to express genes cloned at the *Eco*RI site.

The construction of pAB524 in a similar manner to pAB224 gives rise to a plasmid with two *Eco*RI, two *Hpa*II and two *Hha*I sites, but no *Xba*I site. A potentially useful deletion plasmid would be that formed by circularization of *Eco*RI fragments A plus B of pAB124, which would retain the single *Xba*I site and also contain one *Hha*I site. However, repeated ligations of *Eco*RI-digested recombinants of pUB1654 containing *Eco*RI fragments A plus B failed to generate this plasmid; only pAB224 (*Eco*RI A) and pUB1654 containing *Eco*RI fragment A of pAB124 were obtained. This was rather surprising since one would expect circularization of *Eco*RI fragments A plus B to be about as efficient as circularization of *Eco*RI fragments A plus C (pAB524; Table 4).

We intend to examine the ability of pAB124 and pAB224 to express chromosomal functions cloned at the *Xba*I and *Eco*RI sites, respectively, in order to evaluate their use as vectors for gene cloning in *Bacillus* species.

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1: L09136. pUC18c cloning ve...[gi:209210]

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  AUTHORS   Gilbert,W.
  TITLE     Obtained from VecBase 3.0
  JOURNAL   Unpublished (1991)
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            Gilbert under the auspices of the GenBank Currator Program. pUC18c
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               #journal Gene (1983) 26: 101-106
            REFERENCE
               #number 2
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                 238- 396   1461-1303 (c) Lac-Operon
                 399- 455     57-   1 (c) polylinker of M13mp18
                 456- 682   1297-1071 (c) Lac-Operon
                 683-2686   2352-4355 pBR322
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               mut 2243  T  C  3912   pBR322
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               #indicator beta-galactosidase
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      181 accatatgcg gtgtgaaata ccgcacagat gcgtaaggag aaaataccgc atcaggcgcc
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Revised: July 5, 2002.

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EXHIBIT N

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
E. GREEN *et al.*
Serial No. 09/754,083
Filed: January 5, 2001

Group Art Unit: 1652
Examiner: M. N. RAO
Atty. Docket No. 000487.00007

For: **ETHANOL PRODUCTION**

DECLARATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

We, Edward Green, Muhammad Javed, and Namdar Baghaei-Yazdi, declare that:

1. We are the named inventors of the subject matter claimed in the above-identified application. We deposited Bacillus strains LN (accession number 41038), TN (accession number 41039), LN-S (J8) (accession number 41040), and LN-D (accession number 41041) with the National Collections of Industrial, Food and Marine Bacteria (NCIMB) at 23 St. Machar Drive, Aberdeen AB2 1RY, Scotland, United Kingdom. Each deposit was made under the terms of the Budapest treaty.

2. Upon issuance of a patent each of these strains will be irrevocably and without restriction or conditions be available to the public.

3. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these were made with the knowledge that false statements made willfully are punishable by fine, imprisonment, or both a fine and imprisonment under Section 1001 of Title 18 of the United States; and further that false statements made willfully may jeopardize the validity of any patent issuing on an application in which the false statements were made.

20/01/03
Date

Edward Green

17/01/03
Date

Muhammad Javed

17/1/03
Date

Namdar Baghaei
Namdar Baghaei-Yazdi

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FEMS Microbiology Letters 26 (1985) 333–336
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Mutants of *Bacillus stearothermophilus* lacking NAD-linked L-lactate dehydrogenase

(Fermentation; ethanol production; fluoropyruvate resistance; thermophile)

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1. SUMMARY

Bacillus stearothermophilus produces low levels of ethanol following fermentation of glucose at temperatures in excess of 60°C. A major by-product is L-lactic acid. To improve ethanol yields mutants of this organism were isolated which lacked NAD-linked L-lactate dehydrogenase, the enzyme responsible for lactic acid production. This was achieved by selection for resistance to a suicide substrate, fluoropyruvate. Such mutants no longer produced lactic acid and yields of ethanol from glucose were increased by at least a factor of two.

2. INTRODUCTION

B. stearothermophilus is a facultatively anaerobic thermophilic bacillus capable of growing either oxidatively or fermentatively on glucose [1]. During fermentative growth in rich broth with glucose or sucrose as the major carbon source *B. stearothermophilus* displays a "mixed-acid" type fermentation with the production of lactic, acetic and formic acids and some ethanol [2].

The oxidation and fermentative metabolism of glucose by wild-type *B. stearothermophilus* (NCA1503) has been described previously [3,4]. It appears similar to that typified by the enteric bacterium *Escherichia coli* in that under aerobic, oxidative conditions, pyruvate formed by glycolysis is further metabolised by a pyruvate dehydrogenase (PDH) complex [5] to form acetyl CoA, which would normally be oxidised through the citric acid cycle. Growth under fermentative conditions, however, induces the synthesis of a pyruvate formate lyase (PFL) system [6] which converts the pyruvate to acetyl CoA, but with the concomitant production not of NADH as with the PDH complex but of formate. It has been reported previously both for *B. stearothermophilus* itself [2] and for other "mixed-acid" type fermentative microorganisms such as *E. coli* [7] that the pH of a fermentation can drastically affect the ratios of the different possible end-products. For *B. stearothermophilus*, there appears to be a relationship whereby the production of low levels of lactic acid as a fermentative end-product facilitates the production of relatively higher amounts of ethanol. This is due, presumably, to the extra availability both of carbon as pyruvate and of reducing equivalents as NADH under such conditions. We have been investigating the effects of a variety of parameters on the overall fermentation balance of

this organism as a model system for the improvement of ethanol-producing thermophiles for possible use in a thermophilic ethanol fermentation process. In order to improve ethanol yields, therefore, we attempted to abolish completely the production of lactic acid during the fermentation of glucose by *B. stearothermophilus* by the manipulation of media and growth conditions, but without total success. We therefore opted to remove NAD-linked lactate dehydrogenase, the enzyme responsible for L-lactate production, by mutagenesis. We describe here the isolation of mutants lacking this enzyme and a preliminary discussion of their fermentation characteristics.

3. MATERIALS AND METHODS

The starting strain of *B. stearothermophilus* used in this study was a prototrophic variant of *B. stearothermophilus* NCA1503. This strain, PSII, was isolated previously as a strain capable of rapid anaerobic growth on glucose (Payton, unpublished). Cultures were grown and maintained on LB glucose medium containing yeast extract (20 $g \cdot l^{-1}$), tryptone (10 $g \cdot l^{-1}$) and D-glucose (2 $g \cdot l^{-1}$) at a final pH of 7.0. For plates, LB glucose medium was solidified with 1.5% (w/v) Difco Bacto Agar. Growth was performed at 60°C unless otherwise stated.

Fluoropyruvic acid (sodium salt) was obtained from Sigma (London). Mutants of *B. stearothermophilus* PSII resistant to this compound were isolated first by growing PSII overnight at 60°C aerobically in LB glucose medium. Samples of 0.1 ml of these cultures containing approximately 10^8 viable cells were then plated onto LB glucose medium solidified with agar and allowed to dry. Fluoropyruvate was then applied, as a solid (2–5 mg) to the centre of each plate and the plates were incubated overnight at 60°C, after which time zones of inhibition of 1–3 cm could be seen within a lawn of bacterial growth. Occasionally single colonies could be seen within the zone of inhibition and these were picked onto LB glucose solid medium for purification. Single colonies purified in this way were retested by repeating the original isolation procedure. Only those strains which exhibited zones of inhibition repeatedly and significantly less than the parental strain PSII (i.e. < 0.5 cm) were chosen for further study.

Initial classification of these strains was performed by growing the strains individually in 2-ml volumes of LB glucose medium in static culture at 60°C for 12 h. Half of the culture was centrifuged at $5000 \times g$ for 5 min and the resultant supernatant assayed for the presence of L-lactic acid using a commercially available kit for the determination of L-lactic acid (Boehringer). The remaining half of the culture was permeabilised by a modification of the method previously published for *E. coli* [8].

To 1 ml samples of culture, 10 μl aliquots of a 10% (v/v) solution of toluene in absolute ethanol were added. Following incubation on ice for 20 min the mixtures were vortexed for 1 min and returned to ice. Samples were used within 30 min of preparation to measure NAD-linked L-lactate dehydrogenase by the method of Tarmy and Kaplan [9].

The assay contained in a total volume of 1 ml: NADH (0.3 mM), sodium pyruvate (30 mM) in potassium phosphate buffer (0.1 M, pH 7.5). Sufficient permeabilised extract was added to give an absorbance change of approx. 0.1 min^{-1} at 340 nm and 25°C. Assay results were verified by preparing sonicates of the appropriate strain; cells from 100 ml overnight cultures in LB glucose medium were harvested by centrifugation, washed twice in 10 ml volumes of potassium phosphate buffer (0.1 M, pH 7.5) and finally resuspended in 5 ml of the same buffer. Suspensions were sonicated for 5 1-min bursts (150 W MSE ultrasonic disintegrator, peak to peak amplitude 12 μm) at 0°C and centrifuged at 4°C and $12000 \times g$ for 15 min to remove debris. Lactate dehydrogenase was then assayed in supernatants as described above.

Determinations of the products of glucose fermentation were performed on supernatants from cultures grown on either CG medium (tryptone 30 $g \cdot l^{-1}$, yeast extract 10 $g \cdot l^{-1}$, D-glucose 10 $g \cdot l^{-1}$) or SG medium (tryptone 1 $g \cdot l^{-1}$, yeast extract 0.5 $\cdot$ $g\ l^{-1}$, D-glucose 10 $g \cdot l^{-1}$) for 40 h at 57°C. Assays for lactic acid were performed as described

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above. Ethanol and acetate were also measured using commercially available kits from Sigma (U.K.) and Boehringer, respectively.

4. RESULTS AND DISCUSSION

We had previously failed to isolate lactate dehydrogenase-deficient strains by conventional techniques such as screening for low acid-producing mutants of *B. stearothermophilus* PSII following growth on glucose either aerobically or anaerobically. We therefore sought an alternative method based on a selection for resistance to a suicide substrate, fluoropyruvate. We had previously noted that the pyruvate analogue fluoropyruvate (FCH_2–CO–COOH) was toxic to *B. stearothermophilus*. The toxicity of fluoropyruvate could depend on its ability itself to inhibit a key metabolic step, or could depend on its conversion initially either to fluoroacetyl CoA or to fluorolactate. In each case, amongst fluoropyruvate-resistant strains, some should be defective in uptake or accumulation of the toxic analogue. In addition, however, mutants should be isolated which affect the target reaction of inhibition or the metabolism of fluoropyruvate to fluoroacetyl CoA (via PDH or PFL) or to fluorolactate (via NAD-linked ʟ-lactate dehydrogenase).

We therefore isolated fluoropyruvate-resistant mutants (MATERIALS AND METHODS) and proceeded to characterise the resulting strains. Strains chosen for further study, designated FPY (fluoropyruvate-resistant) mutants, were analysed by screening glucose-grown cells for the presence or absence of NAD-linked ʟ-lactate dehydrogenase. This was tested initially on cells permeabilised with toluene, then verified using cell extracts prepared by sonication (MATERIALS AND METHODS). The supernatants from the same cultures were also assayed for the presence of ʟ-lactic acid (MATERIALS AND METHODS). The results of these analyses are shown in Table 1. Of 12 strains thus tested 8 failed to produce lactic acid and also lacked detectable LDH activity in permeabilised cell suspensions. Assays performed on sonicates of selected strains were used to verify the absence of LDH. One of these strains, FPY 15, was chosen for further study and was redesignated *Ild*15 (ʟ-lactate dehydrogenase-deficient) and its fermentation balance studied.

Table 1

An analysis of fluoropyruvate-resistant mutants of *B. stearothermophilus* PSII

Strain No.	Lactate production	LDH detected in toluenised cells	LDH present in sonicates
FPY 4	+	+	+
FPY 5	−	−	−
FPY 6	−	−	−
FPY 7	+	+	+
FPY 8	+	+	NT [a]
FPY 9	+	+	NT
FPY 10	−	Low	NT
FPY 11	−	Low	NT
FPY 12	−	−	NT
FPY 13	−	Low	NT
FPY 14	−	−	NT
FPY 15	−	−	−

[a] NT, not tested.

Table 2 shows a comparison of the major fermentation products from glucose of *Ild*15 vs. its parent PSII. First, in both strains acetate formation is higher on complex medium than on simple medium. We believe this is due to conversion of amino acids in the complex medium into acetate. Second, ethanol production by *Ild*15 is higher by a factor of two than by PSII. We have subsequently performed a number of fermentations on *Ild*15 both in batch and continuous culture at varying pH which have been published elsewhere [3,4] and have improved the ethanol yield by *Ild*15. The figure quoted for PSII of 0.5 mol ethanol per

Table 2

Yields of fermentation products from glucose (mol product/mol glucose) for *B. stearothermophilus* strains PSII and *Ild*15

The media used and assays of products are described in MATERIALS AND METHODS.

Strain	PSII		*Ild*15	
Medium	CG	SG	CG	SG
Lactate	1	1	0.04	0
Acetate	2	0.5	3.38	1
Ethanol	0.5	0.5	1	1
Total	3.5	2.0	4.42	2.0

mol glucose is, however, the highest we have obtained to date; at lower pHs ethanol production drops off sharply. These data show first that mutants of *B. stearothermophilus* lacking NAD-linked lactate dehydrogenase retain the ability to grow on glucose aerobically or anaerobically. Furthermore, the lactate dehydrogenase-deficient (*lld*) strains of *B. stearothermophilus* produce significantly more ethanol than the wild-type strain following fermentation of glucose, verifying the trends shown by McKray and Vaughn [2]. Results from fermentations performed on *lld*15 suggest that the isolation of similar mutants in other organisms of potential use in ethanol fermentations may be a means of increasing ethanol yields. In addition, the isolation of *lld* mutants of *B. stearothermophilus* amongst fluoropyruvate-resistant strains suggests that in this organism one mode of fluoropyruvate toxicity is dependent on its initial conversion to fluorolactate, in contrast to previous reports of the effect of this and similar fluoroanalogues on *E. coli* [10]. We have, in fact, shown (Payton, M.A., unpublished) that fluoropyruvate is a substrate, albeit a poor one, for semipurified LDH from *B. stearothermophilus*, as has been reported for LDHs from other sources [11].

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